# Autoflex Basic User Manual

Last Updated August 26, 2009

# What is the Bruker Autoflex III?

- Time-of-flight mass spectrometer
  - Ions of given same kinetic energy
    - heavy ions travel slower than lighter ones
  - Two modes of operation
    - Linear (for labile molecules or anything bigger than 5kDa)
    - Reflectron (great for small molecules and peptides)
  - Capable of limited MS-MS
    - Instruments in Proteomics R&D Facility are **MUCH** better for MS-MS and $MS^n$
- MALDI/LDI source
  - 384 position target plate (~1 µL spot size)
  - 355 nm frequency tripled Nd:YAG SmartBeam® laser
    - Can photolyze labile groups, e.g. FITC labels
  - Can analyze positive or negative ions (same spot)

# What Samples Can It Run?

- Biopolymers
  - Peptides, proteins, DNA, RNA, oligosaccharides
- Organometallic complexes
  - Organometallic salts work great
- Some synthetic polymers
  - Polypropylene glycol, PAMAM dendrimers
  - Polycyclic aromatic hydrocarbons with TCNQ
- Molecules that photoionize upon irradiation by 355 nm laser
  - Porphyrins
  - Organometallic complexes

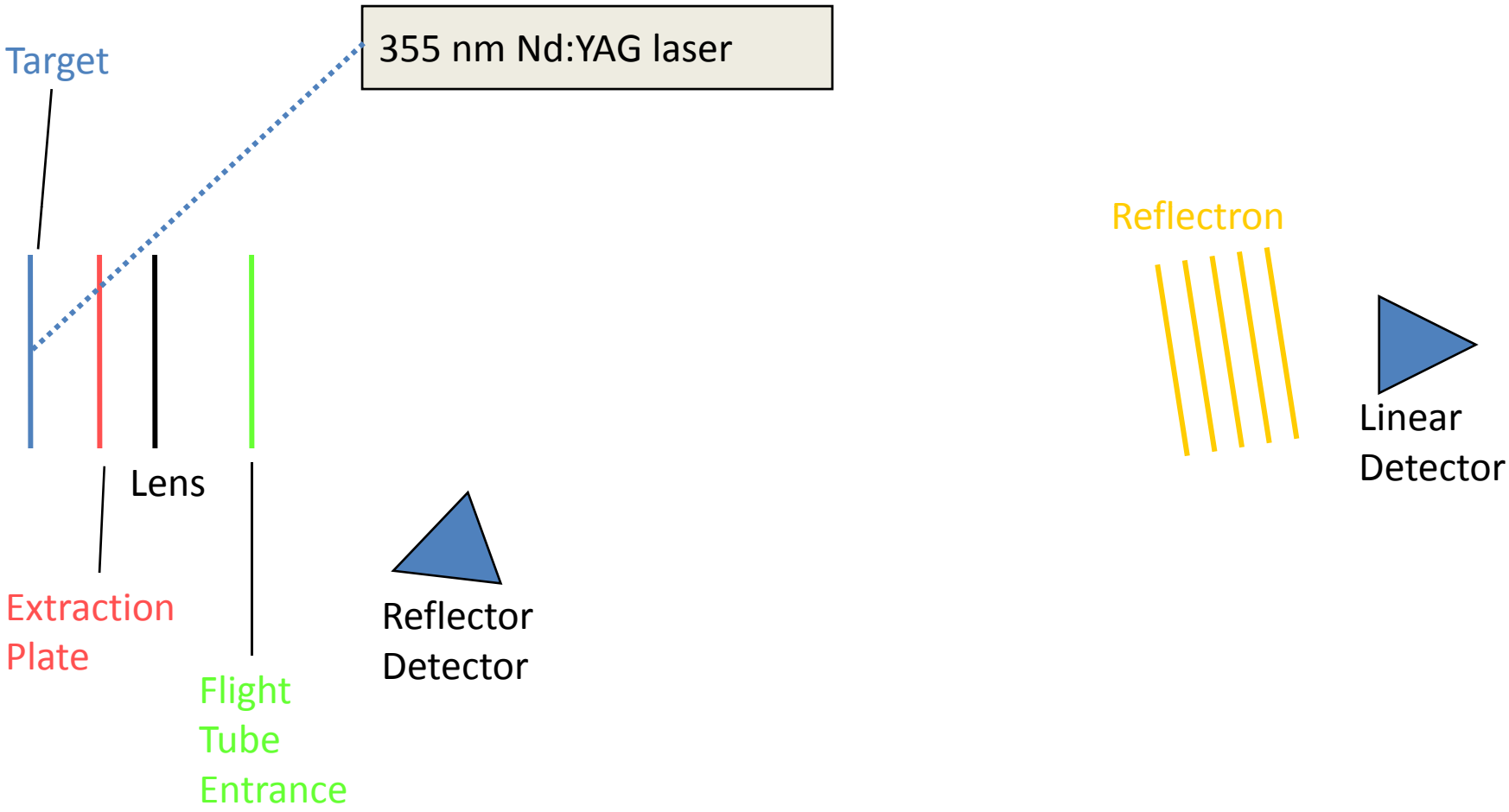
# What Samples Can't It Run?

- "Dirty" samples
  - Significant concentration of involatiles
    - Glycerol, urea, most buffers, many detergents
  - Alkali metal salts can be quite problematic
    - RNA/DNA analyses require extensive desalting
- Molecules with significant vapor pressures
  - Instrument is held at ~$10^{-7}$ torr
- Molecules that  do not ionize in source
  - Lack charge acceptor/donor site
  - Cannot photoionize with $N_2$ laser

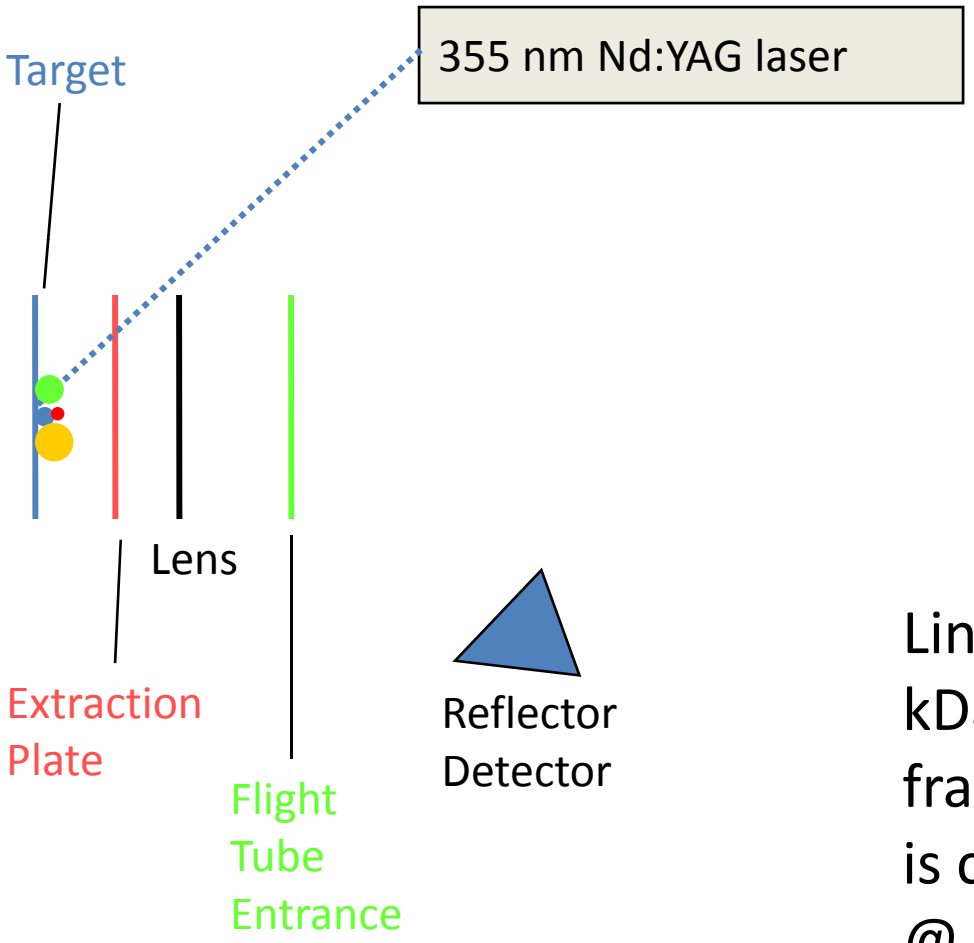
# General Sample Guidelines

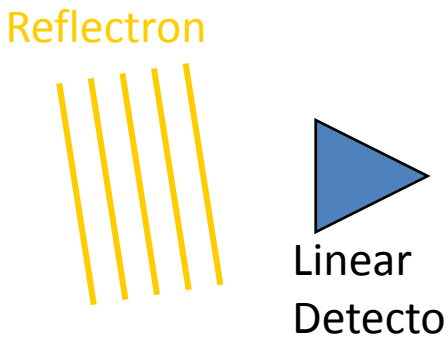
- Purify analyte if possible
  - Analyte should be 5 – 100 µM in concentration
  - ZipTips can purify dirty samples ($C_4$ and $C_{18}$ are in MSF)
- Use only volatile solvents/buffers
  - MeOH, $H_2O$, acetone, $CH_3CN$, THF, $CH_2Cl_2$, $C_6H_6$
  - TFA, HOAc, formic acid, $NH_3$, etc.
  - Ionic strength < 30 mM (e.g. 0.1% v/v HOAc)
- Acidic conditions required for proper crystallization of many matrices
  - Lack of acidic conditions can be overcome in some cases
- Need at least 2 µL

# Instrument Diagram



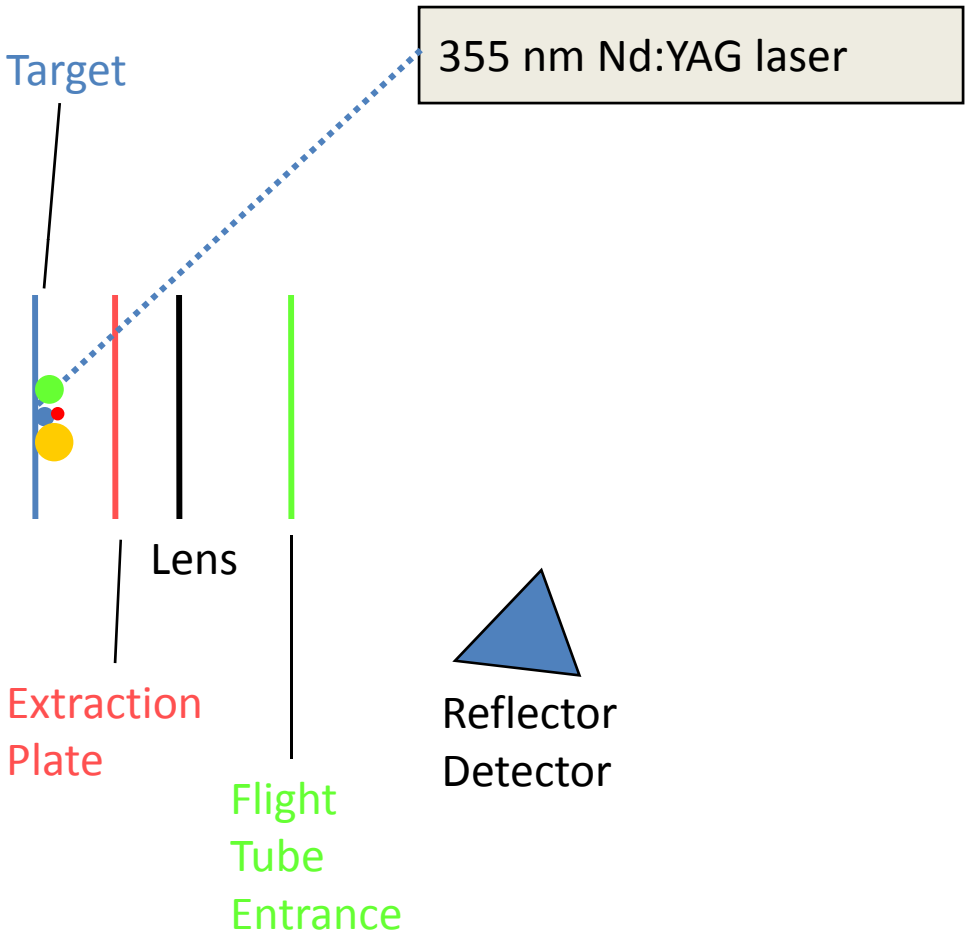
# Linear Mode

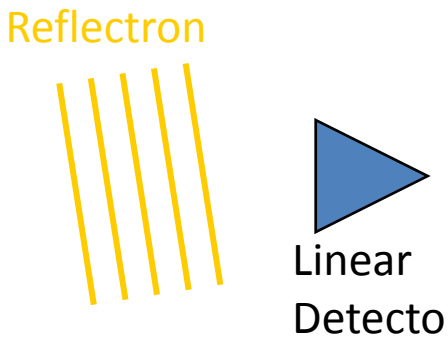



Linear mode is used for large (>3.5 kDa) molecules or exceedingly fragile species (oligosaccharides). It is capable of 4,000 resolving power @ 3.2 kDa (1000 RP @ 12 kDa)

# Reflectron Mode



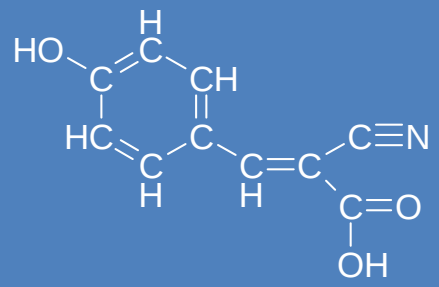




Reflectron mode is used for small species (<3.5 kDa) and is capable of 11,000 resolving power @ 3.2 kDa.

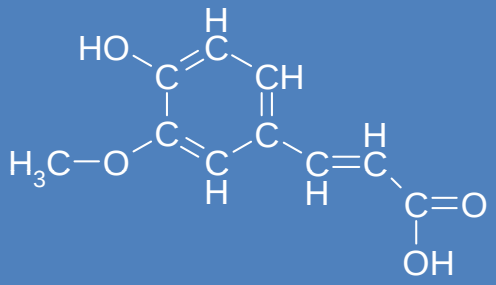
# MALDI Advantages

- Technique is relatively simple
- Volatilize and ionize labile molecules
  - Imagine electron ionization on a protein
- MALDI creates very simple mass spectra
  - Ions are usually $(M+nH)^{n+}$ or $(M-nH)^{n-}$
  - Only 1-3 charge states are observed
    - Usually 1 charge state for peptides < 4 kDa
- MALDI ideal for time-of-flight analyzers
  - Theoretically unlimited mass range (130 kDa done here)
- MALDI is very rapid (<1 min/spot)
- Low sample consumption (1 µL)
- Wide array of matrices available for different analytes

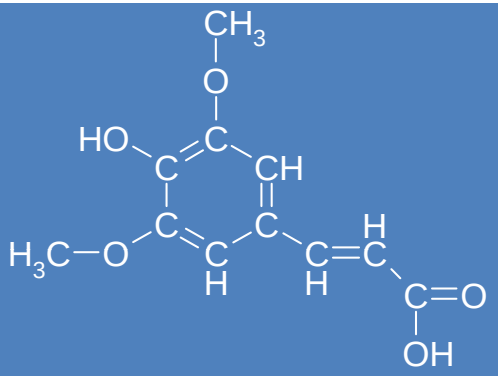
# Some Common MALDI Matrices



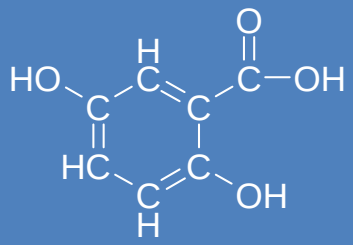
a-cyano-4-hydroxycinnamic acid = CCA
Molecular Formula =$C_{10}H_7NO_3$



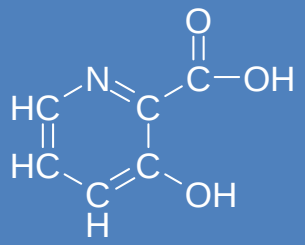
ferulic acid = FA
Molecular Formula =$C_{10}H_{10}O_4$



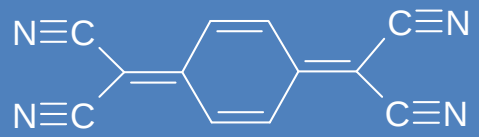
sinapinic acid = SA
Molecular Formula =$C_{11}H_{12}O_5$



2,5 dihydroxybenozoic acid = DHB
Molecular Formula =$C_7H_6O_4$



3-hydroxypicolinic acid = HPA
Molecular Formula =$C_6H_5NO_3$



tetracyanoquinodimethane = TCNQ
Molecular Formula =$C_{12}H_4N_4$

# CCA Matrix

- Good matrix for compounds <10 kDa
  - Makes relatively homogenous flat spots
- Mix 10 g/L in 45% $CH_3CN$, 5% $(CH3)_2CO$, 0.1% TFA in $H_2O$ (instructions on balance)
- Combine 1 part analyte with 5 parts matrix
  - Ratio can be adjusted depending on sample
- Deposit 1 µL on target and let air dry

# FA and SA Matrices

- Matrices for compounds >10 kDA
  - Spots are not homogenous or flat
  - Crystallization often must be assisted (tap spot)
- Sigma premix SA (directions on balance)
  - spots are made by mixing 5:1 matrix:analyte
  - Deposit 1 µL on target (no tapping required)
- Mix 0.15 M matrix in EtOH (alternate method)
  - 32.4 g/L for FA, 33.6 g/L for SA
  - Mix 7 parts analyte + 3 parts matrix; put 1 µL onto target
  - Wait 30 sec
  - Tap spot with pipette tip until tiny crystals form
  - Stir crystals around so entire spot is covered with crystals
- **SA and FA spots require more laser power than CCA**

# HPA Matrix

- Used for oligonucleotides
- 7 mg HPA in 50 µL SCX:$NH4^+$ resin suspension + 50 µL acetonitrile
  - SCX resin desalts matrix and sample
- Deposit 1 µL resin suspension onto target
  - Let resin dry
- Add 1 µL each of analyte and matrix solution to dried resin spot
- Dry sample using heat gun

# DHB Matrix for Saccharides

- Mix up saturated DHB in ethanol
- Mix 1:1 or 1:4 matrix:analyte
- Deposit 1 uL of mixture onto target
- Tap spot for good crystallization (see FA/SA page)
- **DHB spots require much more light than CCA**

# TCNQ matrix for PAHs

- Sample prepared without solvent
- Combine 50-500 parts TCNQ to 1 part sample
- Place mixture and 3 steel BBs into a PCR tube
- Cap tube and tape to vortexer
- Vortex for at least 5 minutes
- Apply small bit of powder to target with the back of a wood Q-tip
- TCNQ requires more light than CCA
  - Ions made by charge exchange, not protonation ($M^{+\cdot}$)

# Mass Axis Calibration

- TOF $\alpha$ $(m/z)^{½}$ is not practical
- $m/z = A*(TOF^2) + B*(TOF) + C$
- Constants are determined by recording mass spectrum of known compounds
  - A variety of calibration mixtures are available
- Instrument can be externally calibrated for quick experiments
- Internal calibration for better mass accuracy can be tricky to perform

# Calibration Part 2

- The Autoflex III is prone to calibration drift
  - Up to 0.25 amu between successive spectra in reflectron mode
  - Up to 2 amu between successive spectra in linear mode
- Instrument should be started at the beginning of each set of experiments (external calibration)
- Calibrant masses need to as precisely defined as possible (3-4 decimal places preferred)
- Calibration can be performed in FlexControl and FlexAnalysis

# Calibration Mixtures

- 4700 mix
  - Peptide mixture with masses from 379-3659
- Mix F
  - Protein mixture with masses from 2466-8566
- Dextran D10
  - Oligosaccharide mixture with masses from 600-2500
- Trypsinogen/Myoglobin
  - Protein mixture with masses from 8,476 to 23,981
- Other compounds and mixtures are available, just ask Jon and Angie

# External Calibration

- Calibrants and anlayte are in different MALDI spots
- Vials of 4700 mix and Protein mix F are in the red MALDI rack.
  - Prepare spots as described previously
  - For other calibrant mixes, talk to MSF staff
- *Make new calibration spots daily*
- Calibration tips
  - Use 4 calibration peaks and a quadratic fit
  - When using mix F, I find using only +1 ions gives better results

# Internal Calibration

- Spot Preparation
  - Mix 1 µL of calibrant mixture with 5 µL matrix
    - This is for mix F and 4700 mix, other mixtures use different ratios
    - Deposit 1 µL on target
  - Mix 1 µL of analyte solution with 5 µL matrix
    - Deposit 1 µL on target (control sample)
  - Mix 1 µL of calibrant/matrix mixture with remaining analyte/matrix mixture
    - Deposit 1 µL on target
- Record mass spectrum and recalibrate the mass scale as described previously
- Multiple tries may be required to find a useful calibrant:analyte ratio

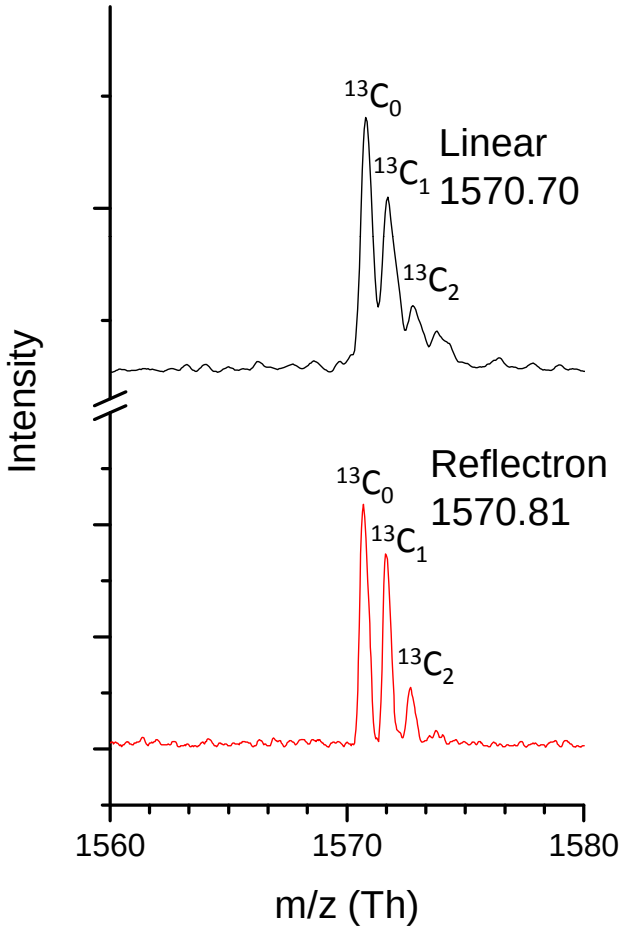
# Common Protein Calibrants

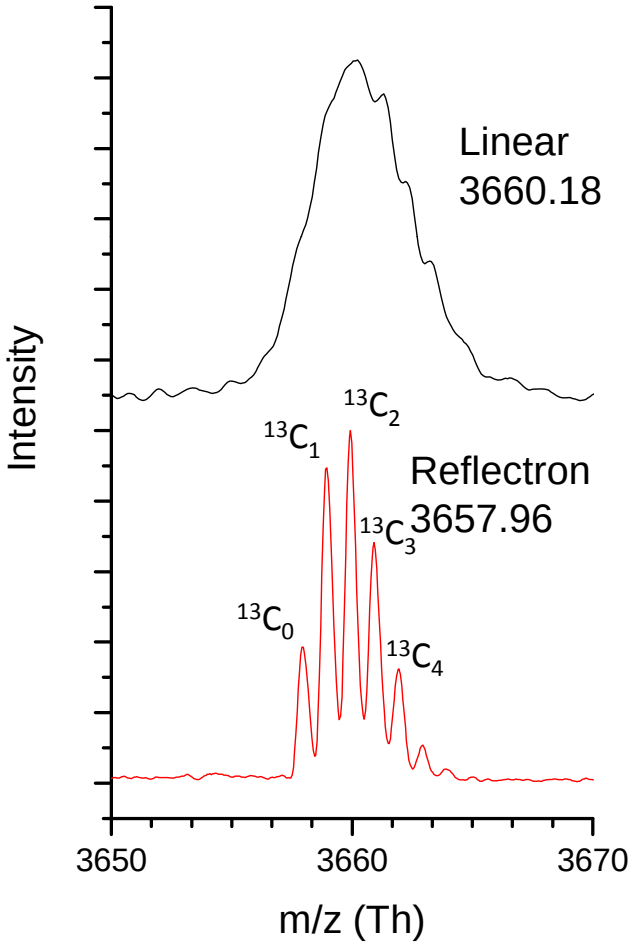
- **CCA_[M+H]+_mono 190.05043**
- **CCA_[2M+H]+_mono 379.09303**
- **heme_mono 616.198**
- **desArgBrady_mono 904.468**
- **Angio_II_[M+H]+_mono 1046.542**
- **Angiotensin_I_M+H+mono 1296.6853**
- **Glu1Fibrinopeptide_mono 1570.6768**
- **ACTH(1-17)_mono 2093.086**
- **ACTH(18-39)_[M+H]+_mono 2465.199**
- **ACTH(18-39)_[M+H]+_avg 2466.68**
- **ubiquitin_[M+3H]3+ 2855.923**
- **Ins_bov_[M+2H]2+_avg 2867.782**
- **Somat_28_[M+H]+_mono 3147.4714**
- **Somat_28_[M+H]+_mono+1 3148.472**
- **Somat_28_[M+H]+_avg 3149.61**
- **ACTH(7-38)_mono 3657.929**
- **ACTH(7-38)_avg 3660.12**
- **Cyt_c_equ_[M+3H]3+ 4121.03**
- **Ubiquitin_[M+2H]2+_avg 4283.446**
- **Ins_bov_[M+H]+_avg 5734.557**
- **Cyt_c_equ_[M+2H]2+ 6181.048**
- **Lysozym_[M+2H]2+ 7153.60**
- **Myo_equ_[M+2H]2+ 8476.780**
- **Ubiquitin_[M+H]+_avg 8565.885**
- **trypsinogen_[M+2H]2+ 11991.47**
- **Cyt_c_equ_[M+H]+ 12361.088**
- **Lysozym_[M+H]+ 14306.20**
- **CarbAnhyd_II_M2H++ 14513.37**
- **Myo_equ_[M+H]+ 16952.551**
- **trypsinogen_[M+H]+ 23981.93**
- **CarbAnhyd_II_MH+ 29025.74**
- **Myo_equ_[2M+H]+ 33904.09**
- **BSA_[M+2H]2+ 33216.**
- **Trypsinogen (2M+H)+ 58049.58**
- **BSA_[M+H]+ 66431.**

# A Word About Isotopes

- Instrument can resolve the isotopic pattern of compounds < 5 kDa in reflectron mode
- Many molecular weight calculators compute the isotopically averaged mass
  - Assume 1.1% of C is $^{13}C$, not $^{12}C$
- Monoisotopic masses are what we label in reflectron mode data
  - All $^{12}C$, $^{1}H$, $^{14}N$, $^{32}S$, $^{16}O$
- Monoisotopic masses are NOT usually observed in linear mode for species > 2.5 kDa
- Be aware of which mass your computer program predicted

# Monoisotopic vs. Average Mass

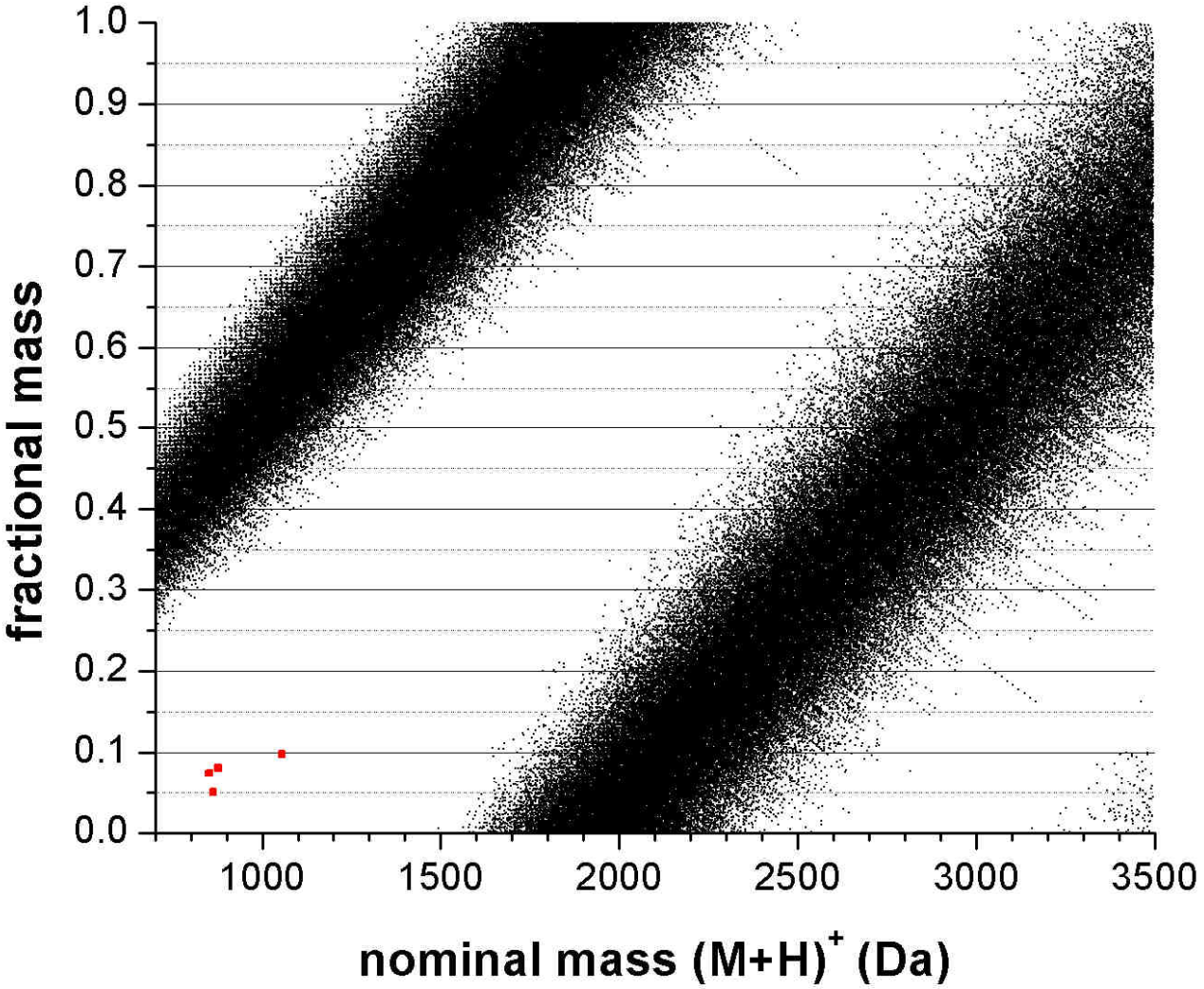


# Interpreting Data: Mass Defect

- **Atomic weights are not integers (except $^{12}C$)**
  - $^{14}N$ = 14.0031 Da; $^{11}B$ = 11.0093 Da; $^{1}H$ = 1.0078 Da
  - $^{16}O$ = 15.9949 Da; $^{19}F$ = 18.9984 Da; $^{56}Fe$ = 55.9349 Da
- Difference from integer mass is called "mass defect" or "fractional mass"
  - Related to nuclear binding energy
- **Sum of the mass defects depends on composition**
  - H, N increase mass defect
    - Hydrogen-rich molecules have high mass defects
    - Eicosane ($C_{20}H_{42}$)= 282.3286
  - O, Cl, F, Na decrease it
    - Hydrogen deficient species have low mass defects
    - Morphine, ($C_{17}H_{19}NO_3$) = 285.1365
- Average peptide: $n*(C_{4.94}H_{7.76}N_{1.36}O_{1.48}S_{0.04})$

# Peptide Mass Defect Figure




**Dependence of the fractional masses of predicted Caulobacter tryptic peptides on their nominal masses. The red squares represent the locations of the matrix-alkali cluster masses**

**[Figure from Karty et al in *J. Chrom. B.* v782 pp363-383 (2002)].**

# Sample Prep Tricks

- Ziptip to clean up dirty samples
  - $C_{18}$ for peptides < 3 kDa
  - $C_4$ for peptides/proteins > 3kDa
  - Elute directly into matrix for added sensitivity
  - ZipTip instructions on MSF website
- If CCA liquid turns yellow, pH is too high
  - Spots from non-acidic CCA do not crystallize correctly
  - Add a little 1% v/v or 10% v/v TFA to lower pH
  - If sample needs base for solubility, try over-layer method
    - Dissolve sample in $NH_3$ or other volatile base
    - Place 1 uL of sample on target, let dry completely
    - Deposit 1 uL matrix over top of dried sample

# Sample Prep Tricks 2

- Non-aqueous over-layer
  - Make 1 uL spot of matrix on plate, let dry
  - Deposit small amount of sample in volatile solvent (e.g. $CHCl_3$, acetone, $CH_2Cl_2$)
  - You can even do internal calibration this way
    - Put calibrants in matrix spot
- For better mass accuracy, let voltages stabilize 5-10 minutes before recording data

# Sample Prep Continued

Samples for MALDI-TOF analysis need to meet certain requirements for obtaining good spectra. The more careful you prepare samples (including early steps of isolation and preparation) the more likely a successful analysis will be. Here are some guidelines of which kind of treatment is advantageous for mass spectrometric analysis and which is not

1. Avoid the use of non-volatile agents like salts (NaCl, CaCl2, KH2PO4), detergents (Tween, Triton, SDS), chaotropic agents (Urea, Guanidinium salts) and non-volatile solvents like DMSO, DMF, or Glycerol.
2. If you can't avoid these agents, purify. Dialysis, ZipTips, and RP-HPLC are good purification methods if you use volatile solvents and buffers (e.g. 0.1% v/v trifluoracetic acid, 10 mM NH4HCO3). After purification, lyophilize if possible. Ion exchange beads may work well for salt removal.
3. Suitable solvents are ones that are volatile. For sample work up and purification: water, ammonium hydrocarbonate, ammonium acetate, ammonium formate, acetonitrile, trifluoroacetic acid.

# Sample Prep 3

4. Quantitate the sample you are going to provide for analysis by methods like: photometry (e.g. OD, Bradford assay), and ELISA. HPLC is useful since it allows for purification and quantitation in a single procedure. The range for many samples/preparations is not very large, therefore it is necessary to have a good estimate of the sample amount because the sample amount may need to be varied on the target.

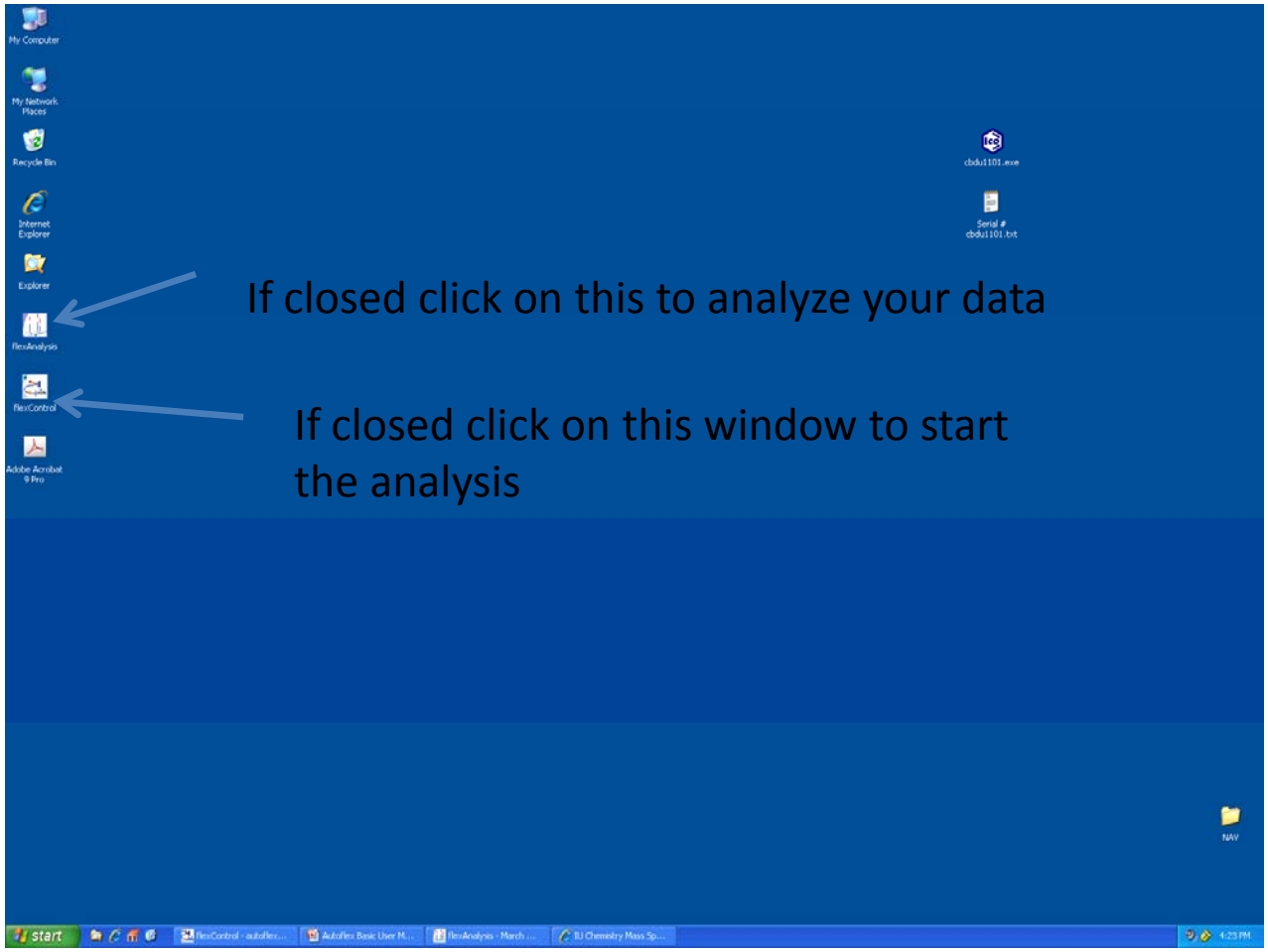
5. The total amount of sample needed for MALDI analysis depends on the sample type. For small mw peptides (1,000 or less) the minimum amount needed for analysis is 16 picomoles/microliter. The minimum for mw 20,000 or less is 60 picomoles/microliter. For 66,000 mw, the minimum amount needed for analysis is 160 picomoles/microliter. Therefore, the larger the molecular weight the more sample is needed.

6. Give information like: structure, sequence, molecular weight, type of compound, biological activity, chemical reactivity, pH, sample amount/concentration, describe purification/isolation with focus on relative agents/solvents, known or suspected impurities, suitable solvents, hazardous properties: radioactivity, carcinogenicity, poison, or explosive.

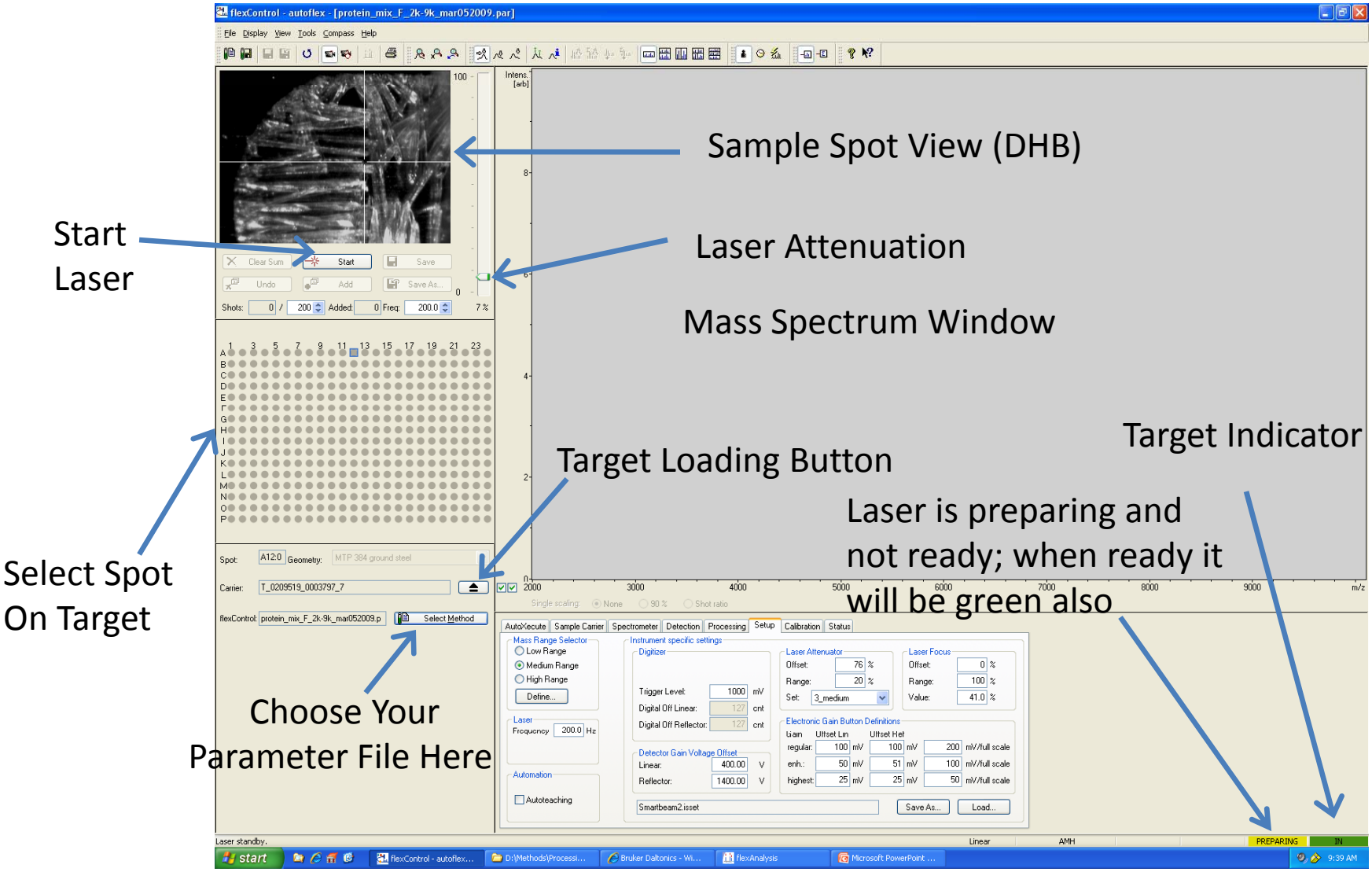
# Maldi on Intact Gel Bands

1. Add 100ul 50uM $NH_4HCO_3$
2. Add 100 ul Acetonitrile
3. Shake for 5 min. on vortexer
4. Discard liquid and repeat 3 more times
5. Add 200 ul HPLC water and let sit 10 min
6. Discard water and and repeat
7. Discard water, add 15 ul matrix; crush gel with tip and let 5 min
8. Spot like normal next to standards and run maldi
9. May not always work, try tryptic digest as an alternative

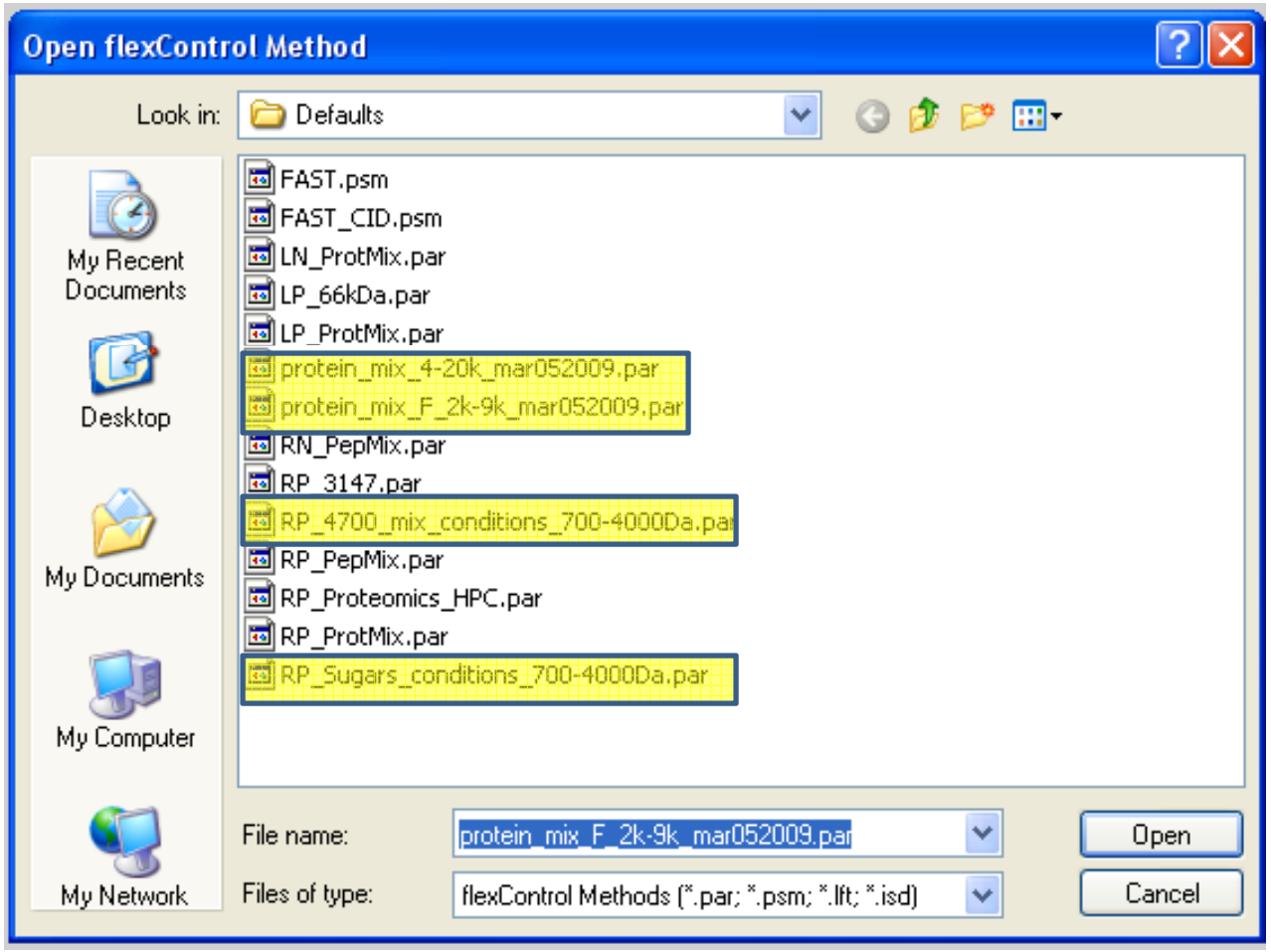
# Starting the Software



# Flex Control Screen


Start Laser
Sample Spot View (DHB)
Laser Attenuation
Mass Spectrum Window
Target Indicator
Target Loading Button
Laser is preparing and not ready; when ready it will be green also
Select Spot On Target
Choose Your Parameter File Here

# Suggested flexControl Methods



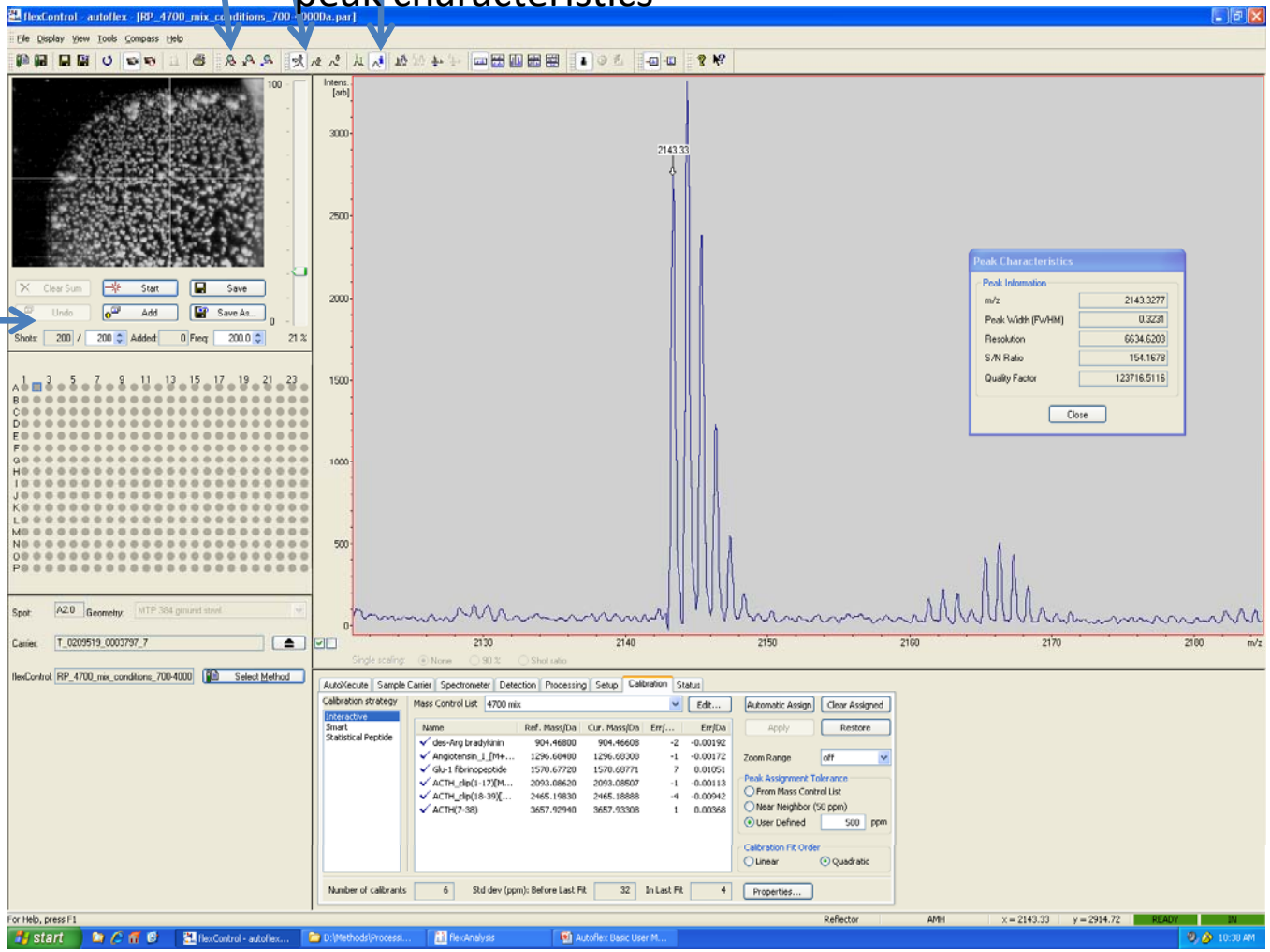

Use the 4 highlighted methods from above

# Acquire Data

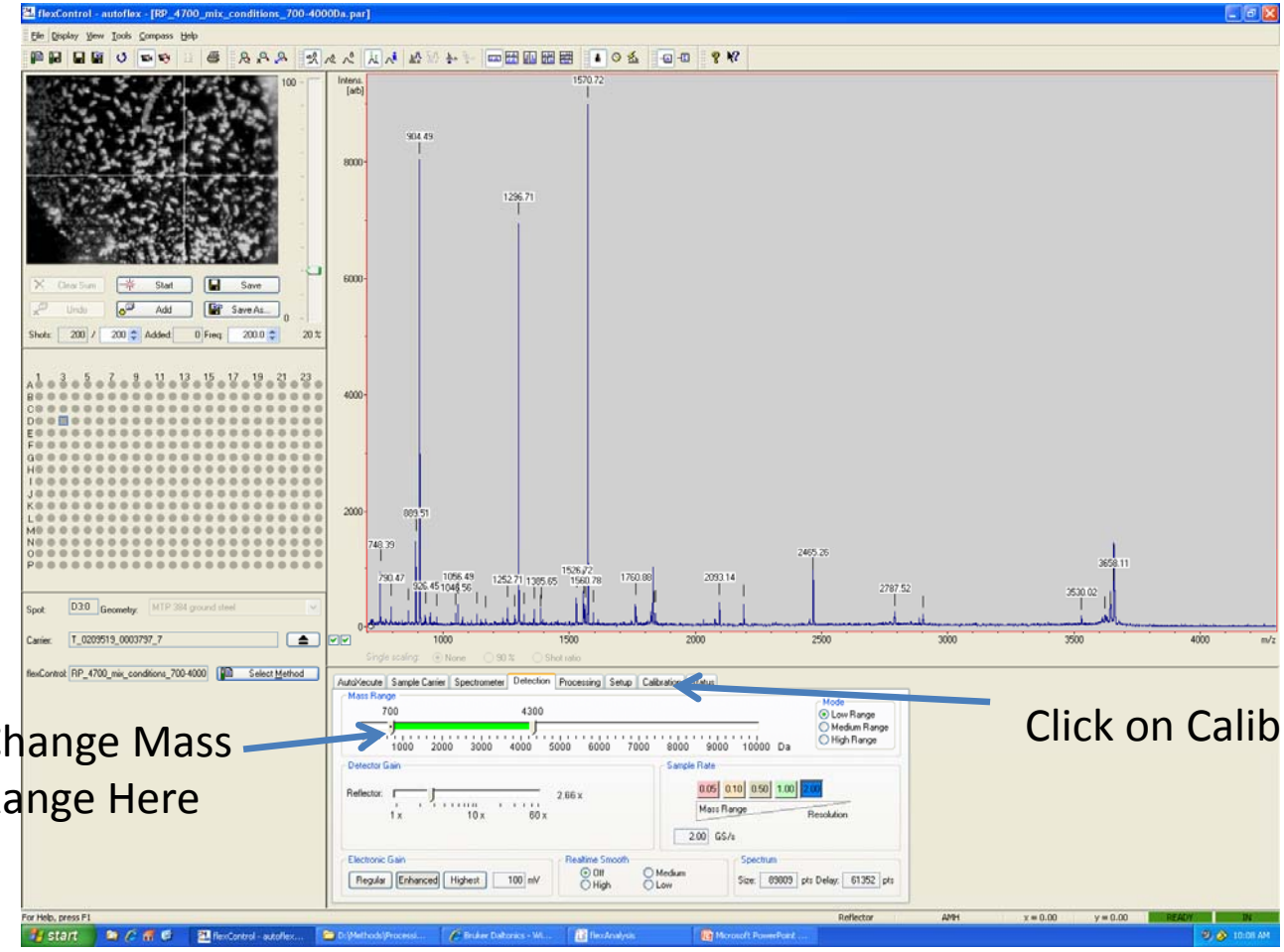
Zoom in on peaks

Clicking max. cursor to left and Peak info. You can get your peak characteristics

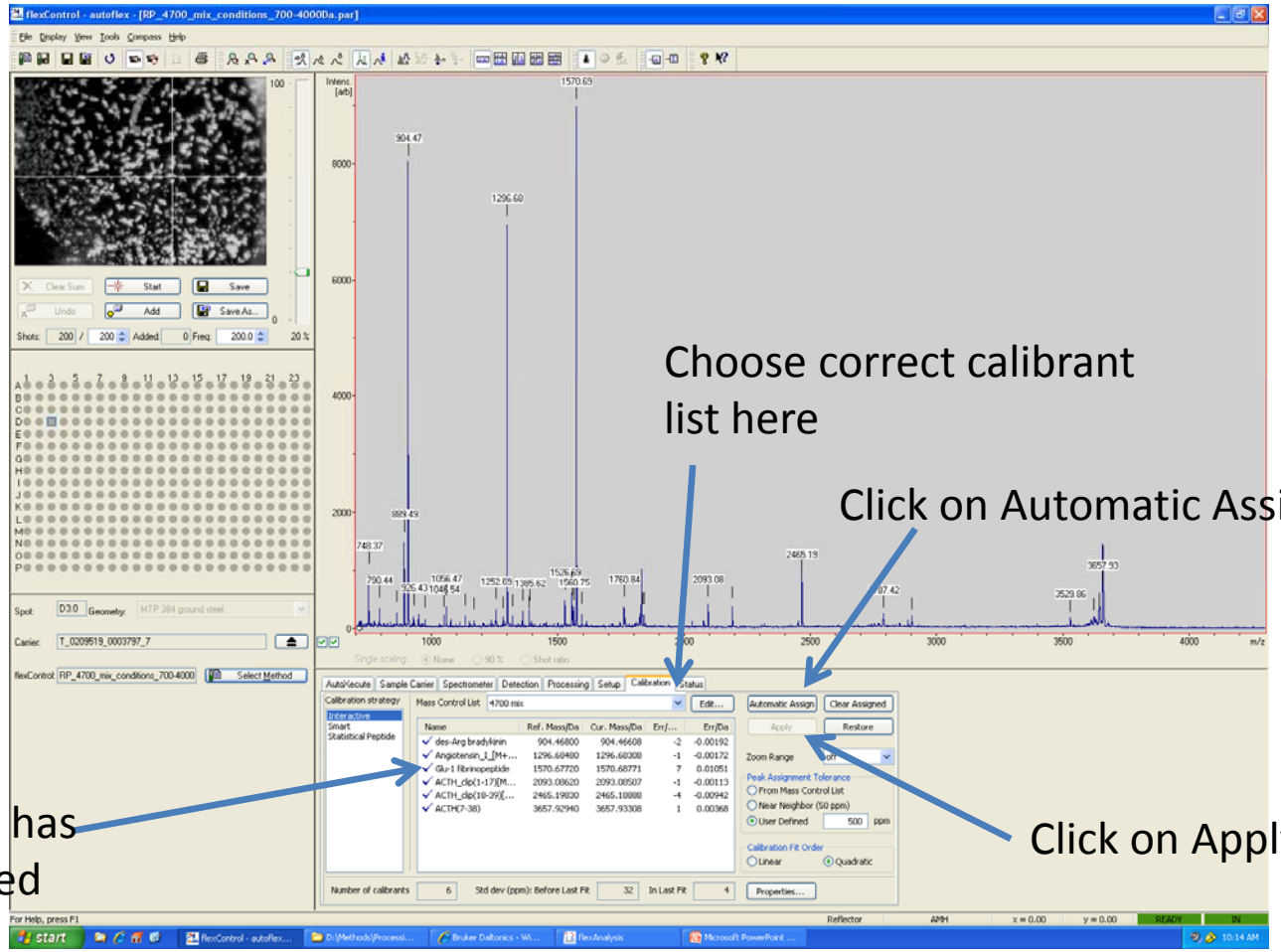
Number of laser shots



# How to Calibrate



# Calibration Continued




All peaks that are found should have a check mark to the left

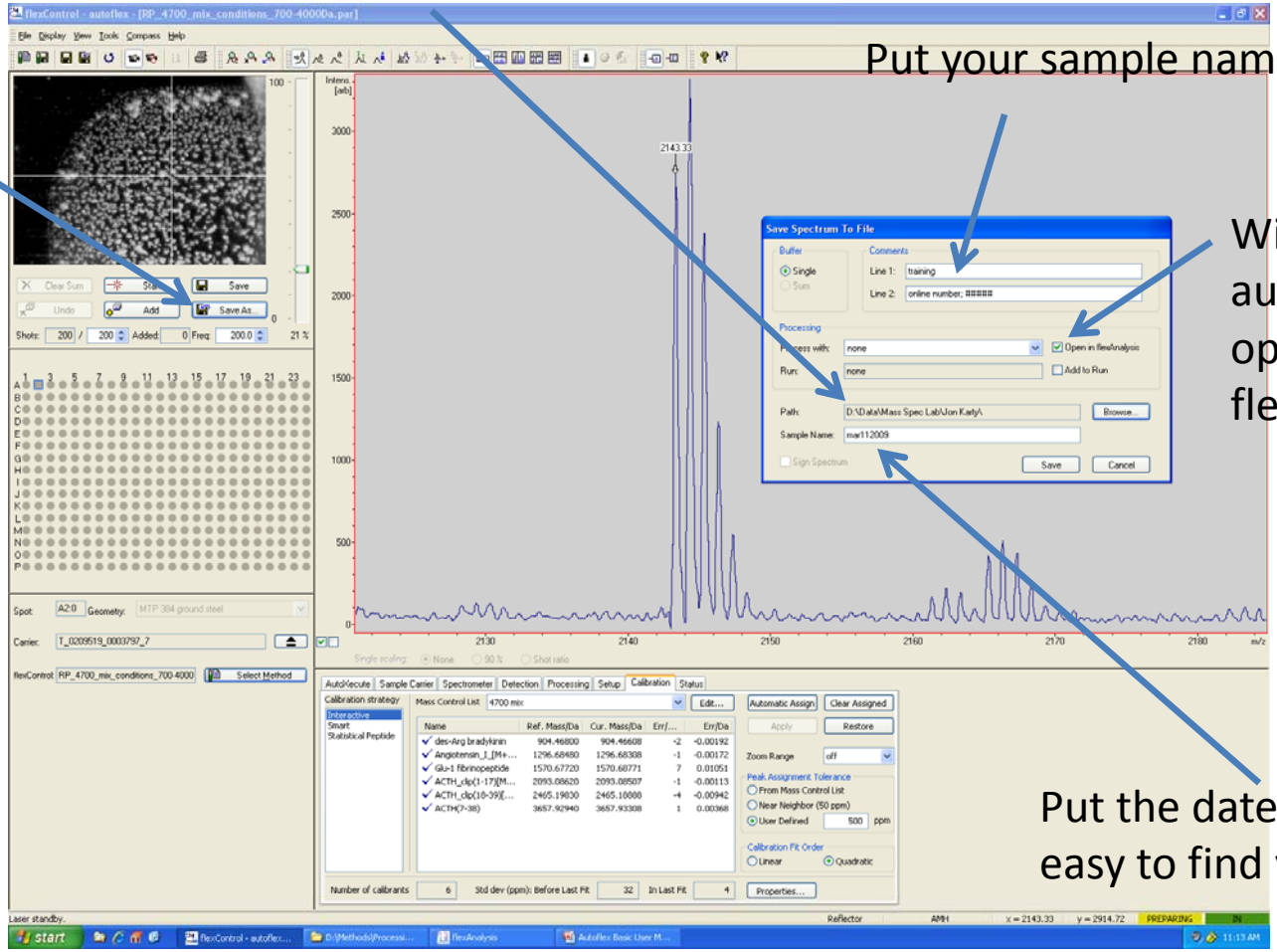
# Calibrant List

- 4700 mix (masses from 904-3660)
- Dextran 680-2350
- Protein Mix F (masses from 2465-8465)
- MSF Big proteins (masses 6181-132861)
- Porphyrins
- 4700 neg (902-3658 negative ion)
- You can make your own lists (see Jon or Angie)

# Saving Data

Data Path should always be:
Data\Group\User

Click the "Save As… button to bring up the "Save Spectrum To File" Window

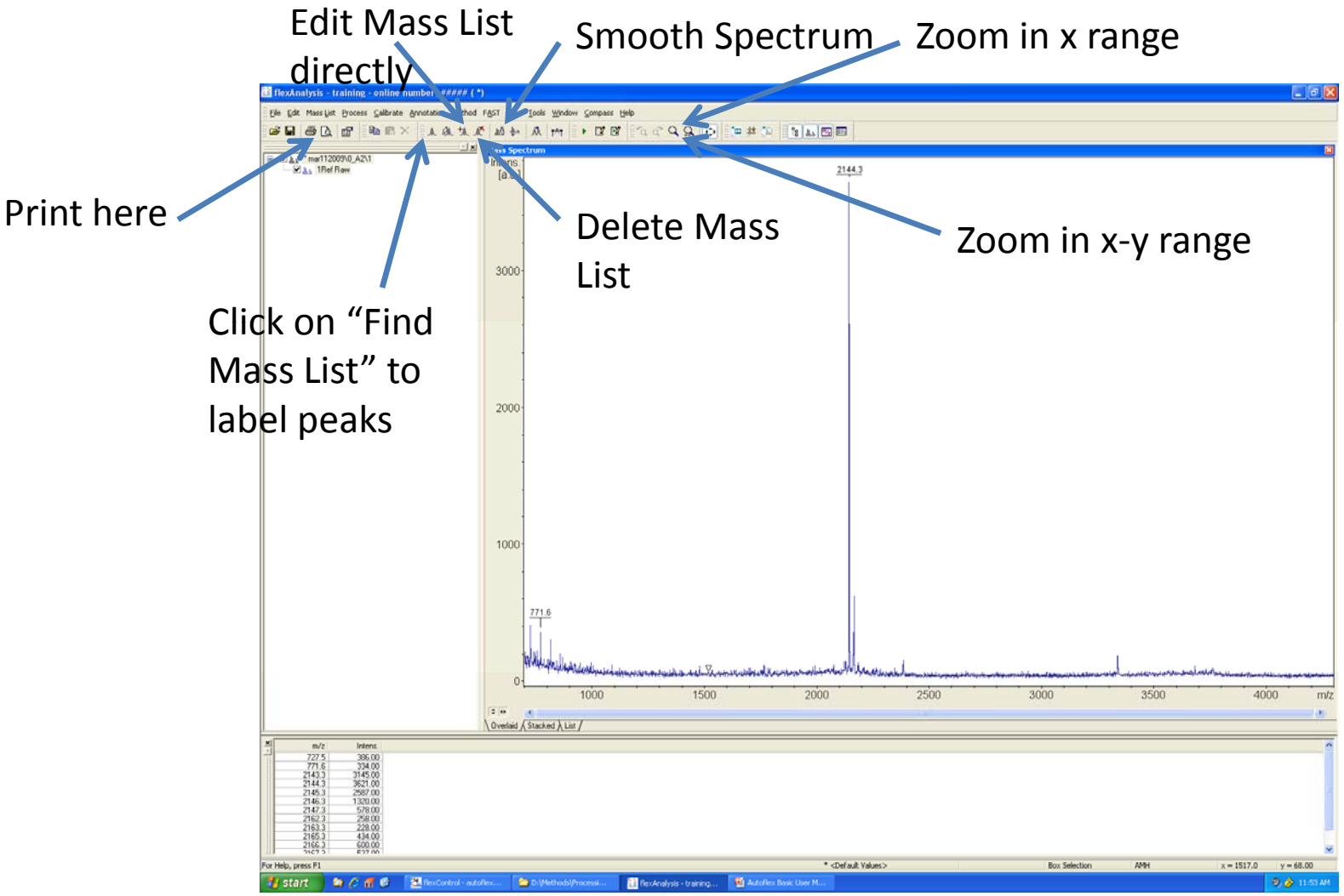



Put your sample name here

Will automatically open in flexAnalysis

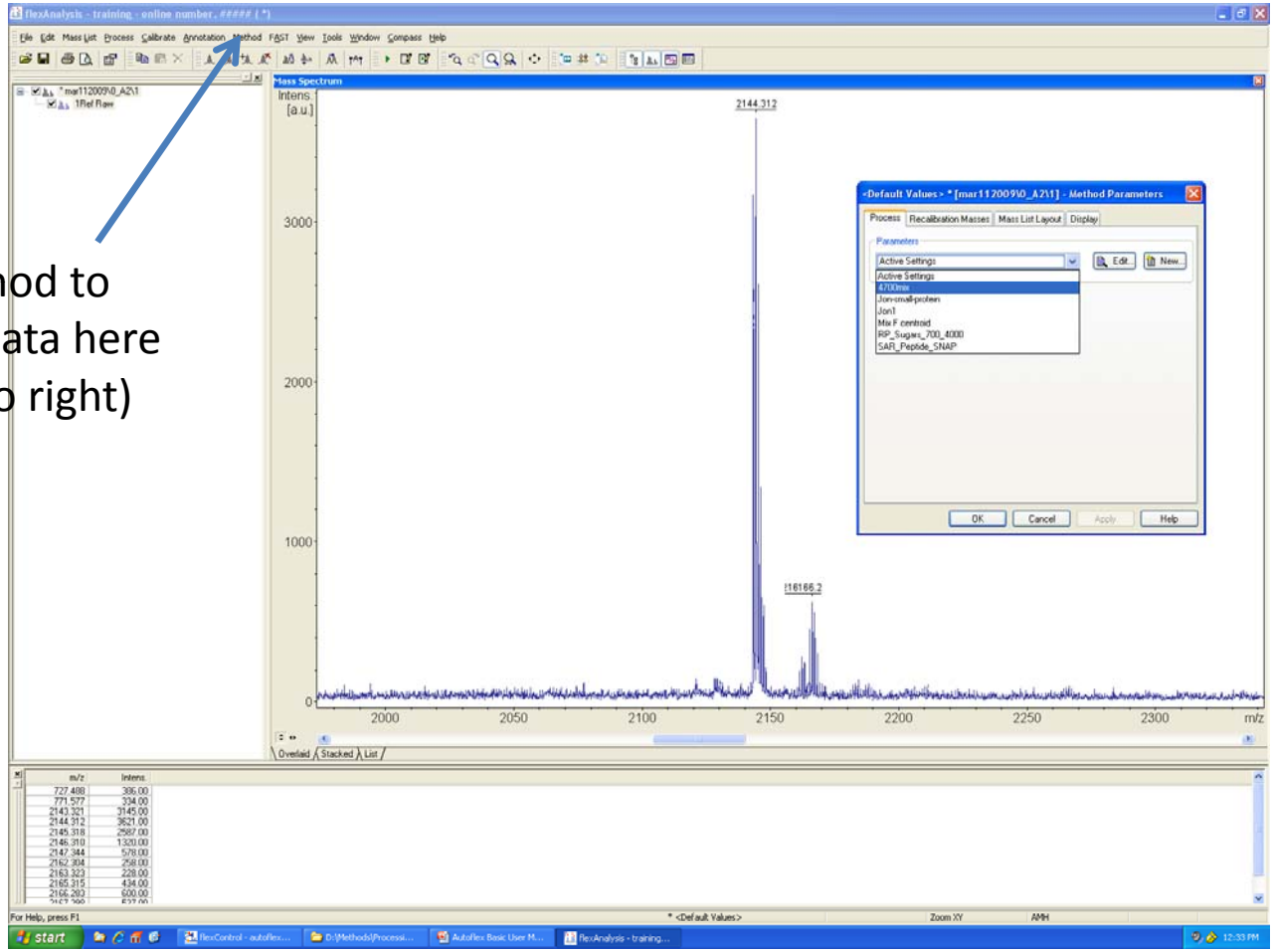
Put the date here so it's easy to find your data

# Looking at Data in flexAnalysis

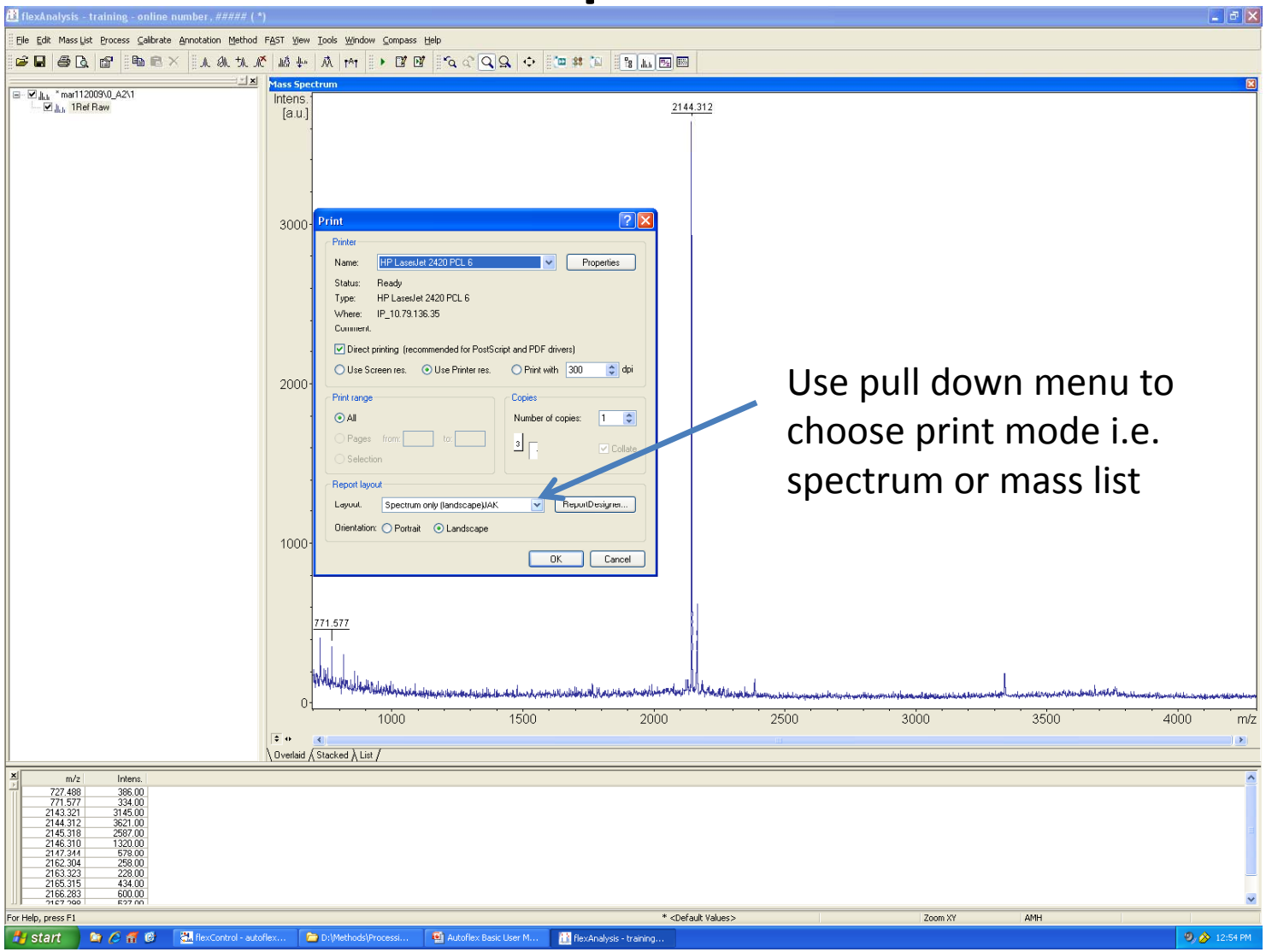


# Processing Data in flexAnalysis

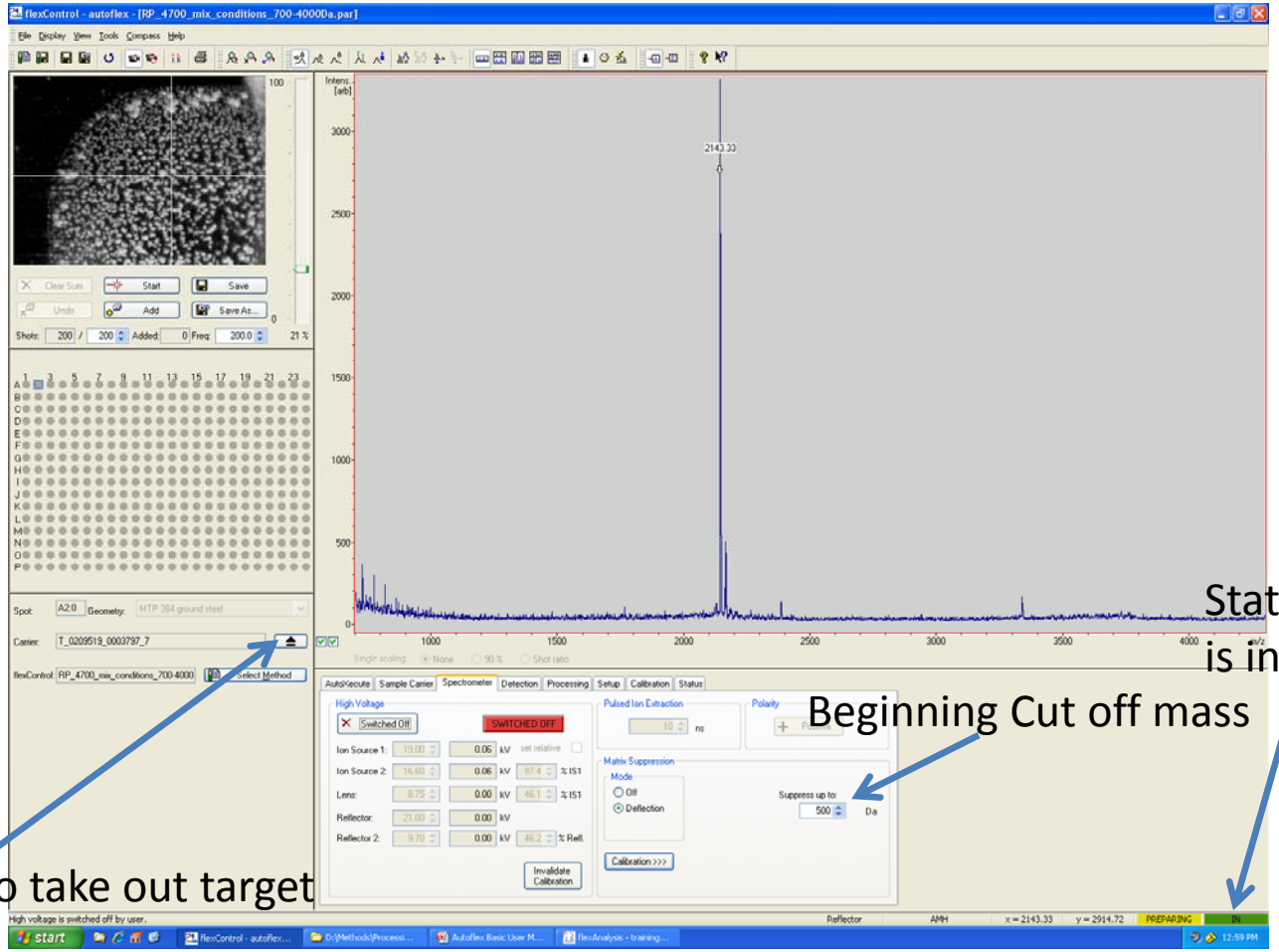
Pick your method to process your data here (see window to right) and apply



# Print Spectrum

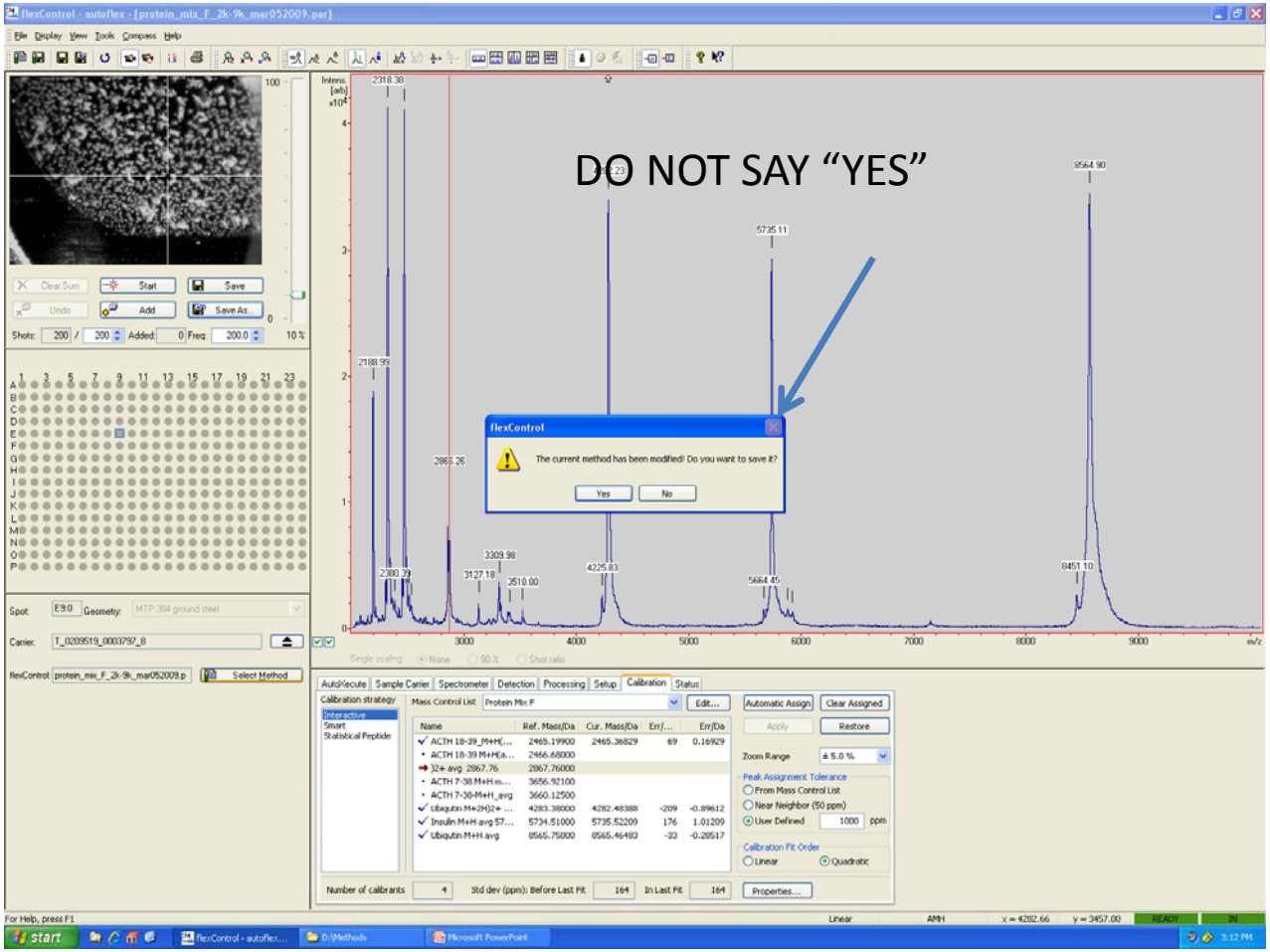


# When Finished




Place target on Maldi prep bench with the plastic cover, and click the target in button once more to close the Autoflex door. Load the RP_4700_mix_conditions_700-4000 so the instrument HV comes on and stabilizes for the next user.

# Note: Never save a change to a method that's been modified!



# Online Billing

- Always enter the samples online first before you run  your sample(s) so you can put your data base number in with your sample name
- Go to http://msf.chem.indiana.edu/default.htm to enter your sample(s)
- Log samples you run on the paper sheet next to the printer